Extended One-Generation Study with Antiandrogens

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One Generation Extension Study

- The study is not one recommended for routine use in a testing hierarchy.
- Hypothesis driven study.
- Should provide valuable information in guiding necessary amendments in protocols for (tailored) Tier 2 testing.
- Use of known positive antiandrogens which work through different pharmacologies to cover a range of end points.

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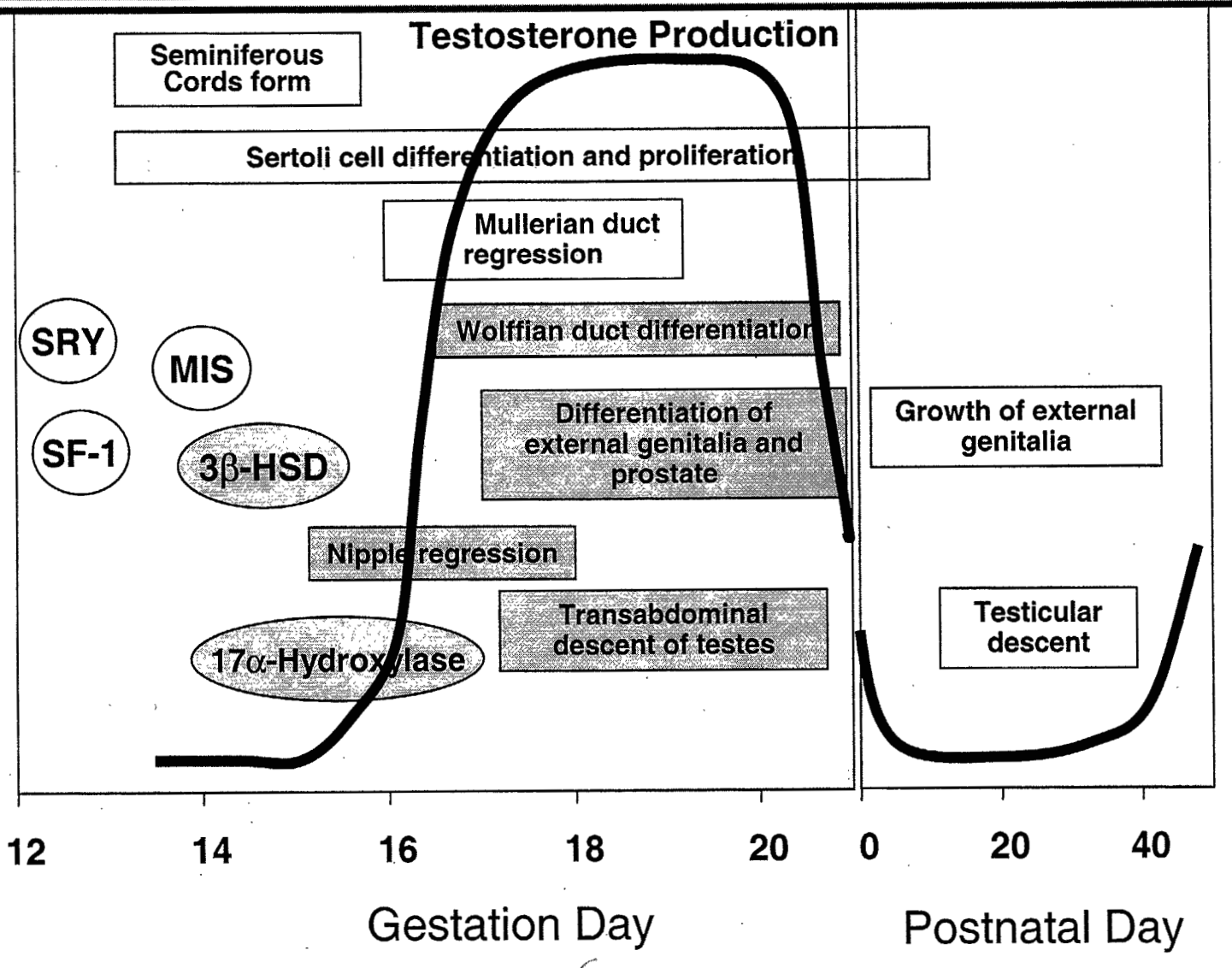
Purpose of Tier 2 test

- Provide "definitive" information on hazard characterization of EACs (EPA).
  - Confirm or refute observations noted in Tier 1 screens/ assays.
  - Utilize *in utero* (most sensitive) exposure.
  - Identify activity vs. end points for which concern has been raised in humans (eg hypospadias, cryptorchidism,↓sperm); identify other endocrine-like activity.
  - Provide dose-response information to be used in risk assessment.

Why this study?

- Covers exposure during critical developmental windows not covered in standard teratology studies or any of the Tier 1 screens and assays.
- Antiandrogens known to produce specific malformations in this critical window based on the knowledge of the biology of reproductive development.

Development of the Rat Male Reproductive Tract



Issues Addressed -1

- Detection of adverse effects.
  - At what age can the full range of compound mediated malformations and changes be observed?
- Dose response relationships.
  - Would the investigation accurately describe and address all adverse changes over a dose range?
- Numbers of animals/ litter employed at different ages.
  - Do we need to keep animals to adulthood and/or can an improved weanling examination and necropsy be made to better characterize effects?

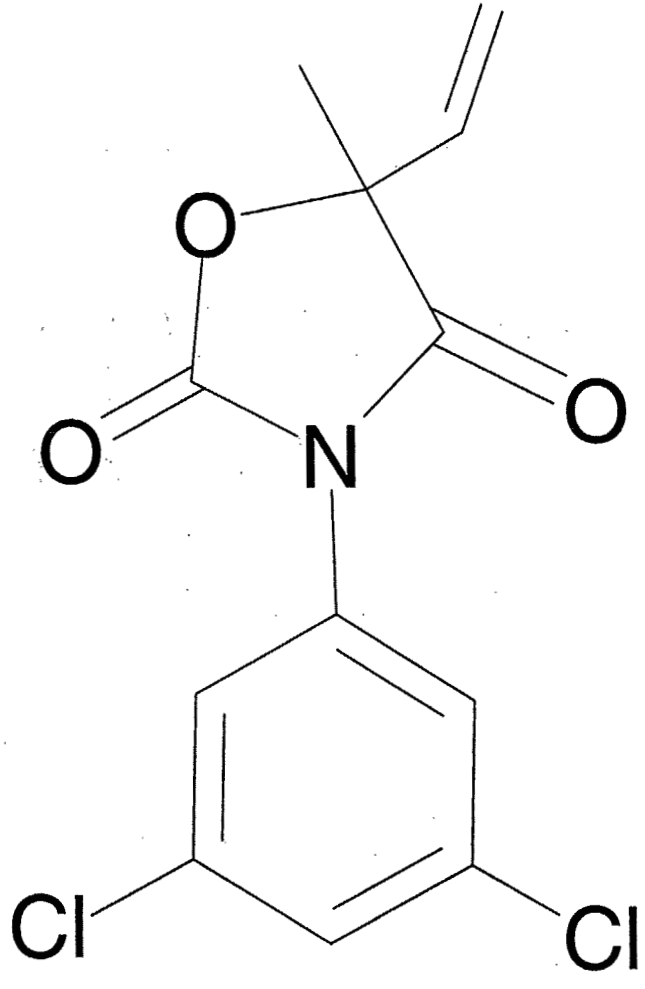
Issues addressed -2

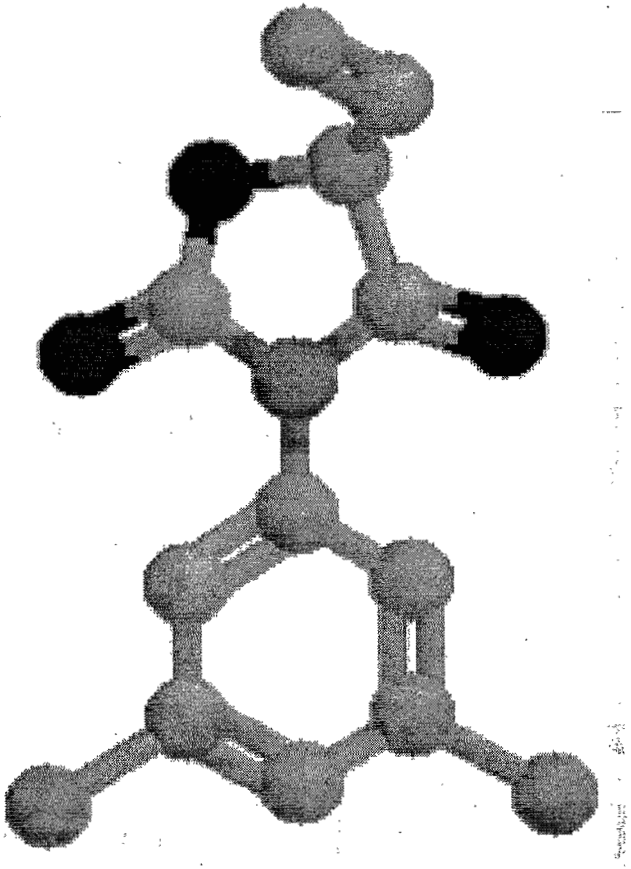
- Can we accurately discriminate between transient and permanent structural changes?
  - AGD and nipples.
- What are the critical end points that need to be included in (tailored) testing protocols to accurately characterize suspected antiandrogens?
- However, this study did not address histopathological issues regarding numbers examined (10 /group versus larger numbers) to accurately characterize biologically and statistically significant changes (eg potential sensitivity of testicular lesions for DBP).

Selection of Test Agents

- Use antiandrogens that have well characterized dose–related responses when administered in an *in utero/* lactational type study with retention of full litter complement.
- Vinclozolin (VIN): a fungicide shown to be an Androgen Receptor antagonist.
- Di-n-butyl phthalate (DBP): a plasticizer and solvent that reduces fetal testicular androgen production.
- Lowest dose level based on measurement of critical end points (particularly hypospadias and prostate for VIN and AGD/ nipples, testis for DBP).

Structure of Vinclozolin





Critical End Points for Vinclozolin

- Hypospadias
- Prostate agenesis/ malformations
- Vaginal pouch

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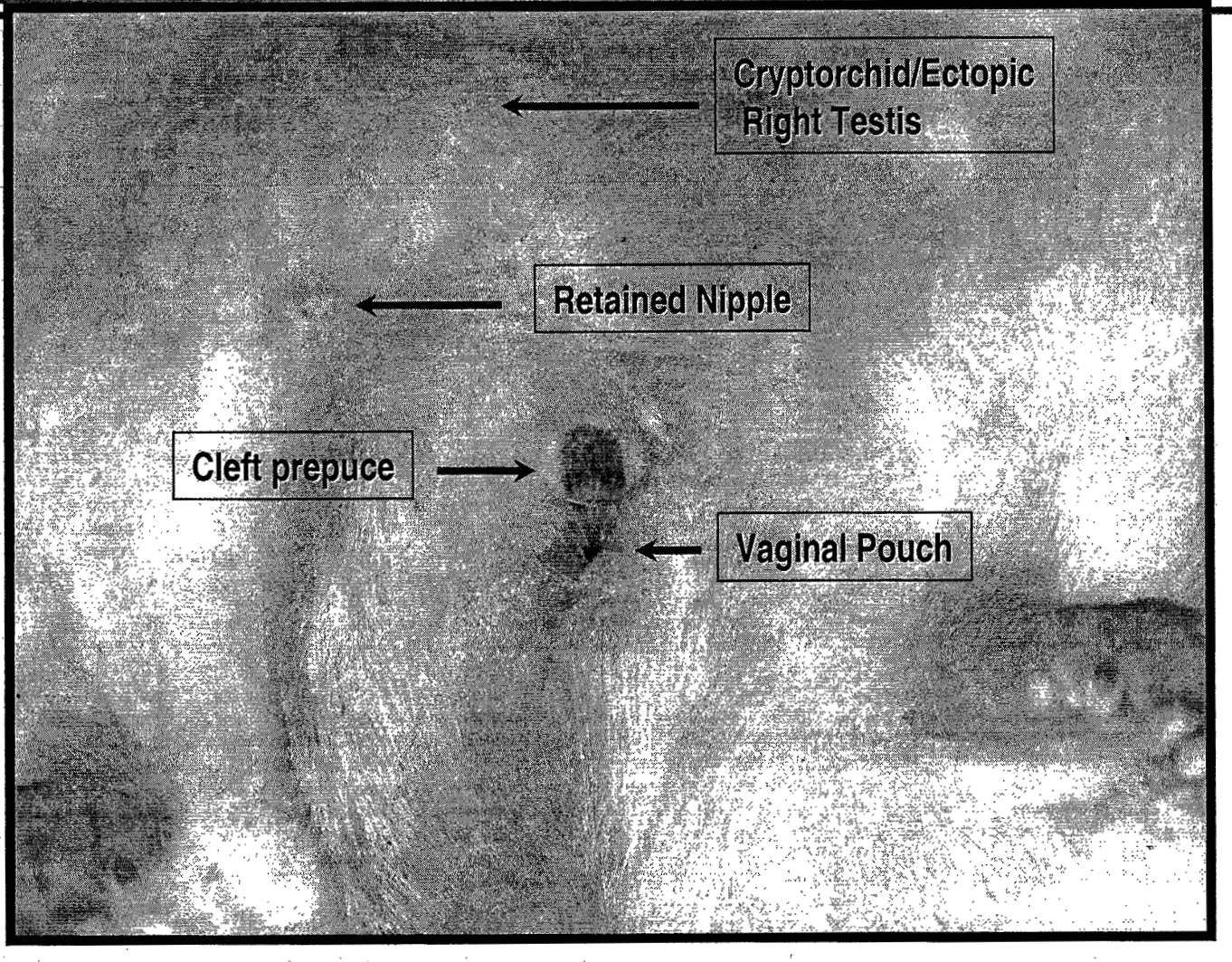
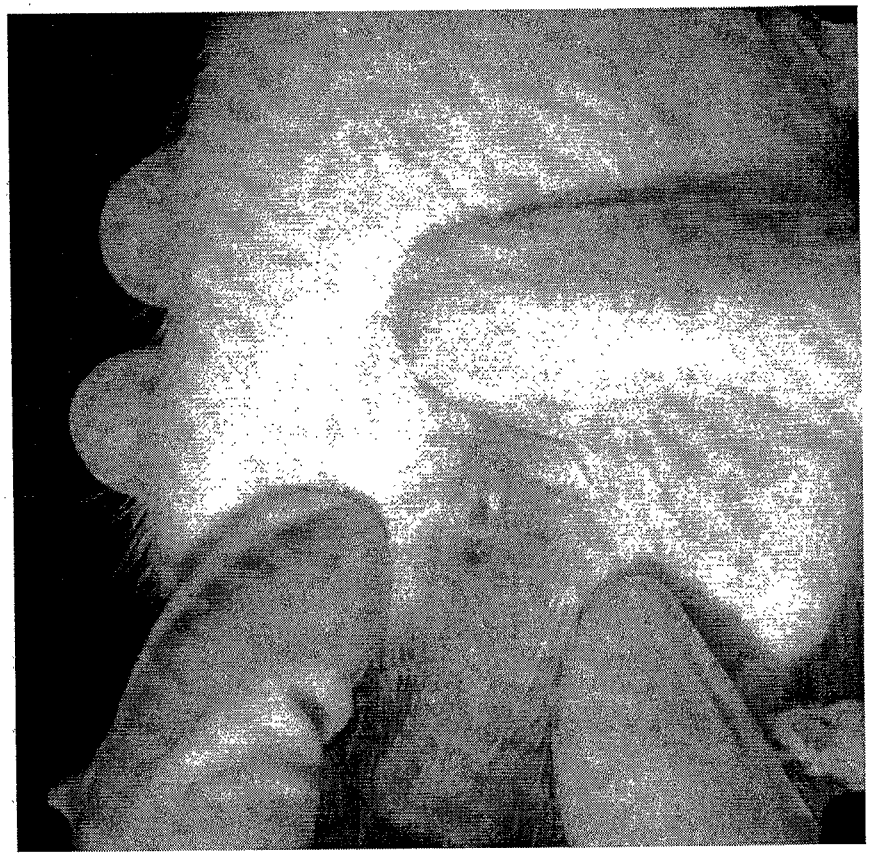
External male reproductive tract malformations



Hypospadias and vaginal pouch following Vinclozolin administration



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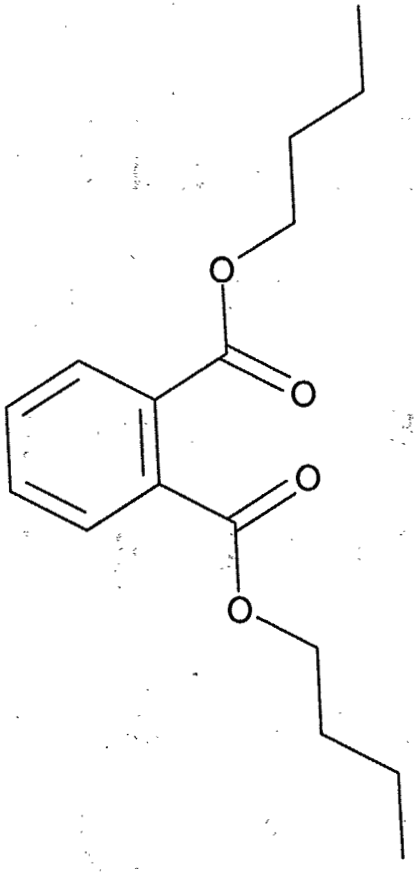
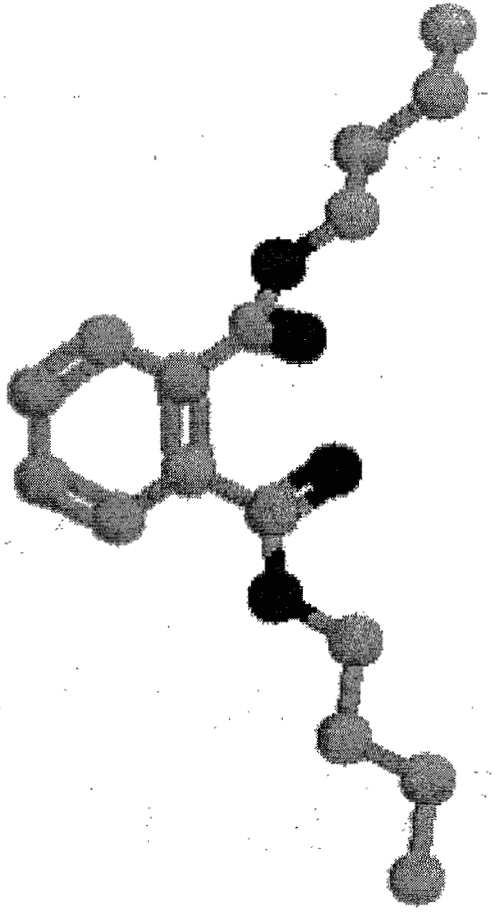
Di-*n*-Butyl Phthalate





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Critical End Points for DBP

- Epididymal malformations
- Hypospadias
- Testicular effects
- Permanent changes in AGD and nipples

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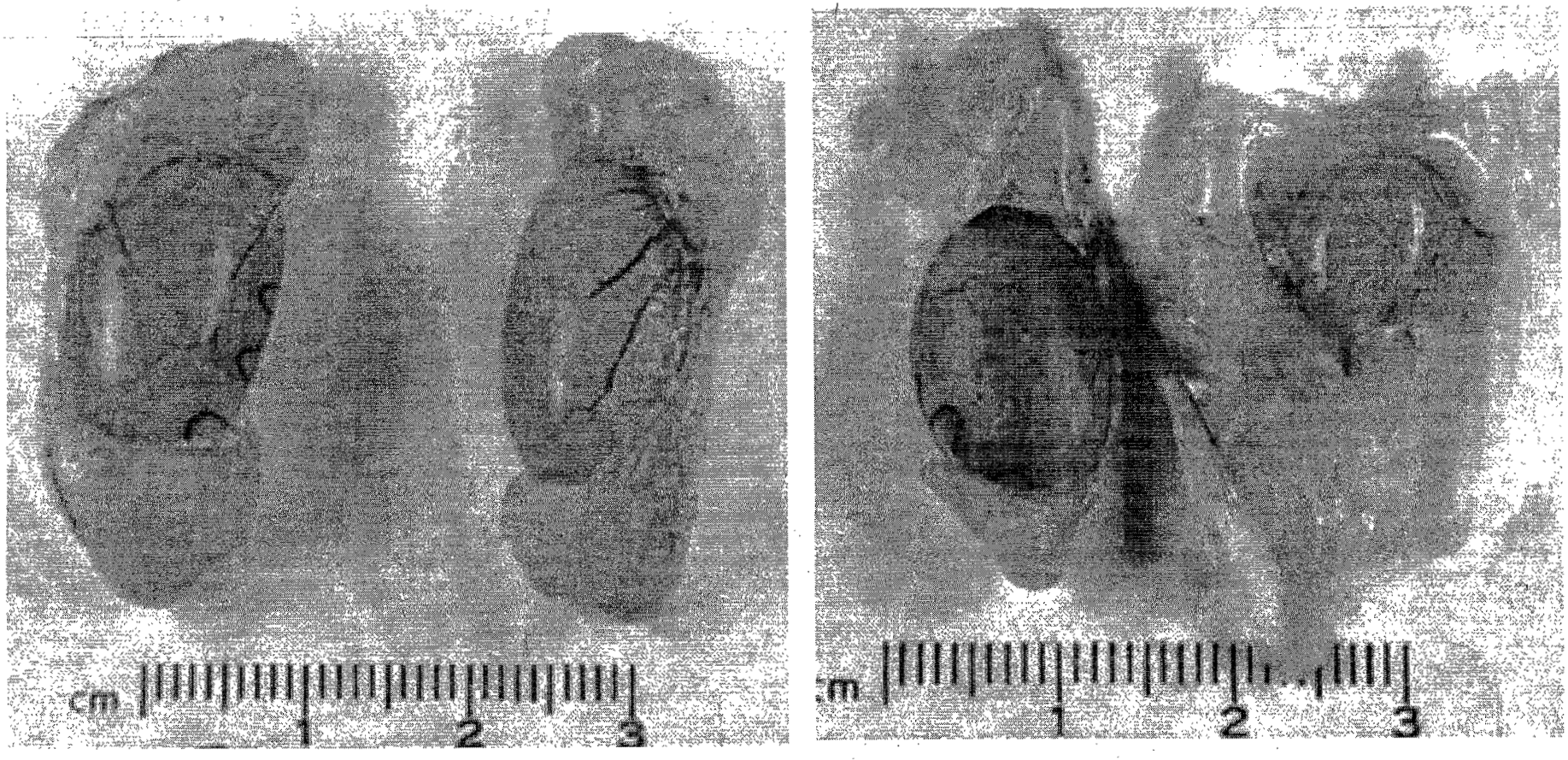
Testis & Epididymis following *in utero* DBP exposure

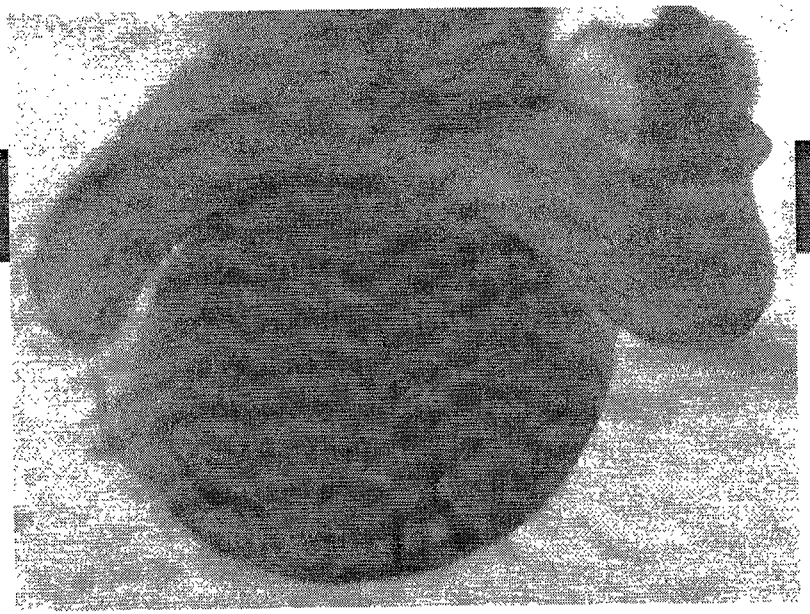
Control

DBP

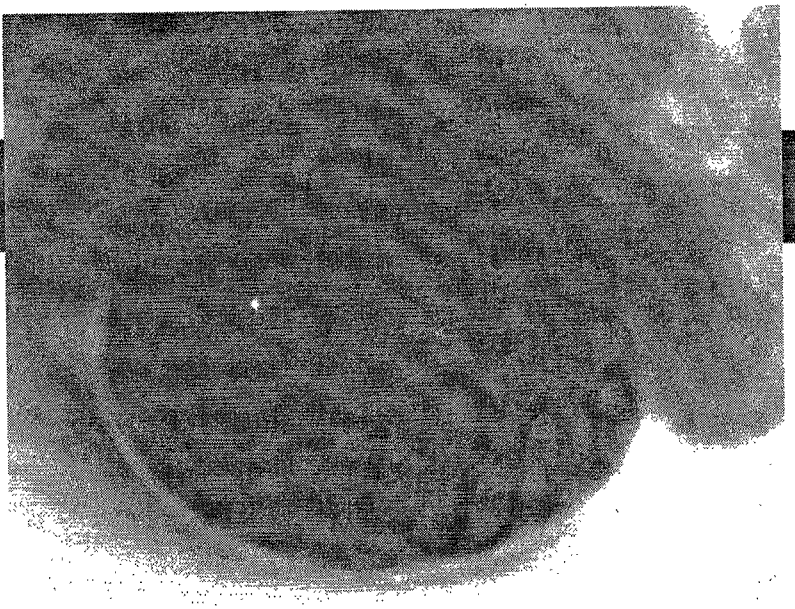


Mylchreest *et al* (1998), *Tox Sci* **43**:47-60

GD 19 control

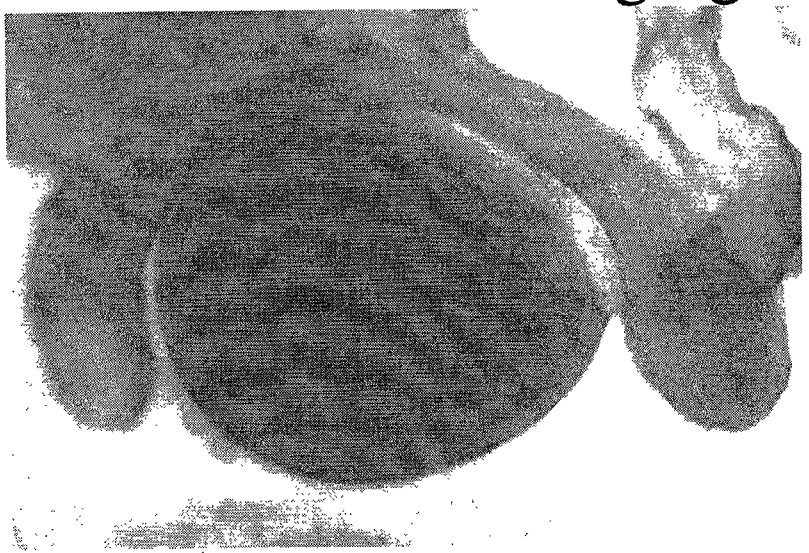


GD 19 DBP 500mg/kg



GD 21 control

GD 21 DBP 500mg/kg



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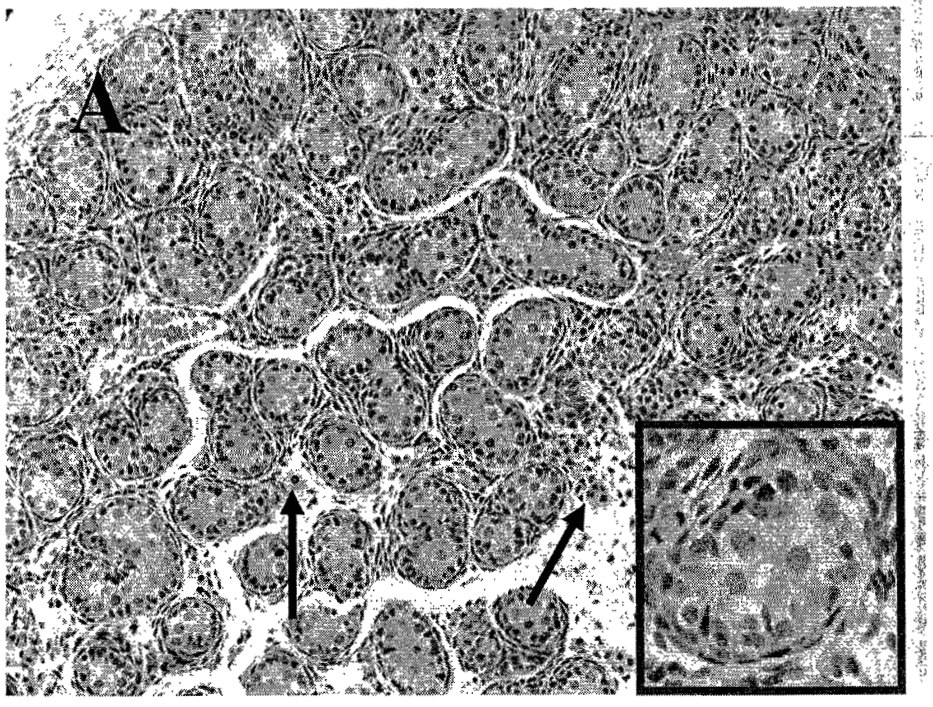
Interstitial Cell Hyperplasia and Multinucleate Gonocytes after DBP treatment - GD 21



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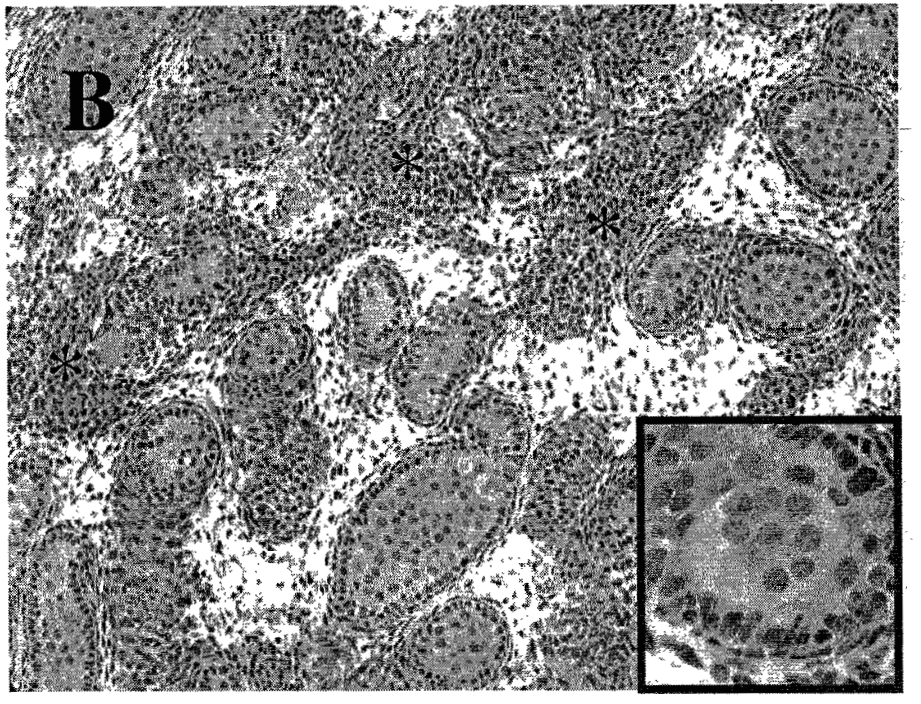

DBP-treated

Barlow et al (2003) *Tox Sci* **73**: 431-451

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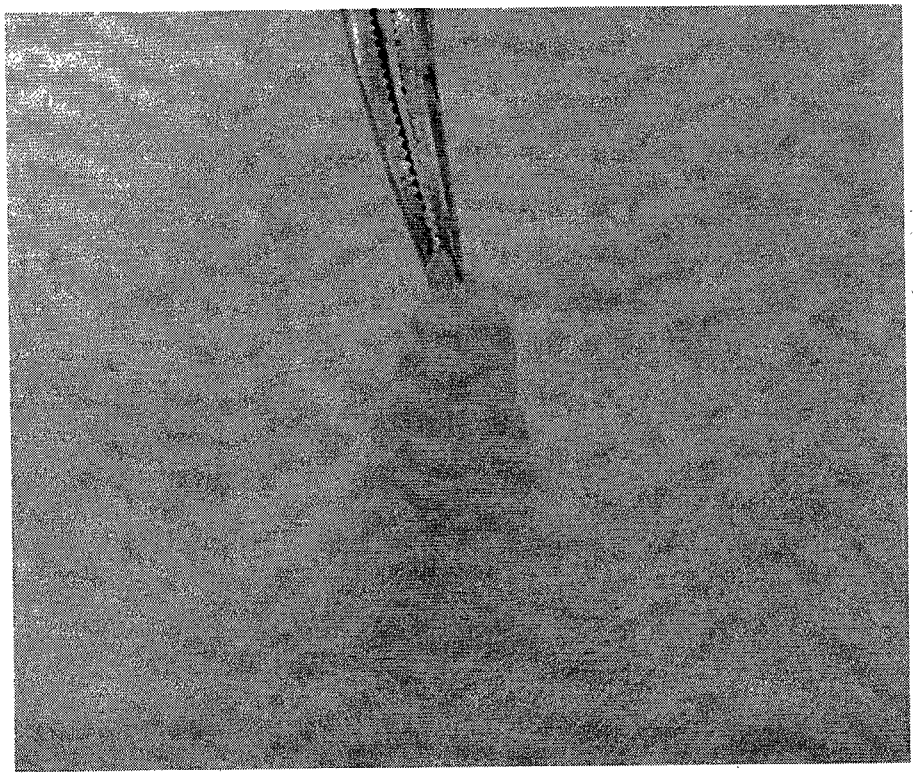
DBP-Induced Hypospadias



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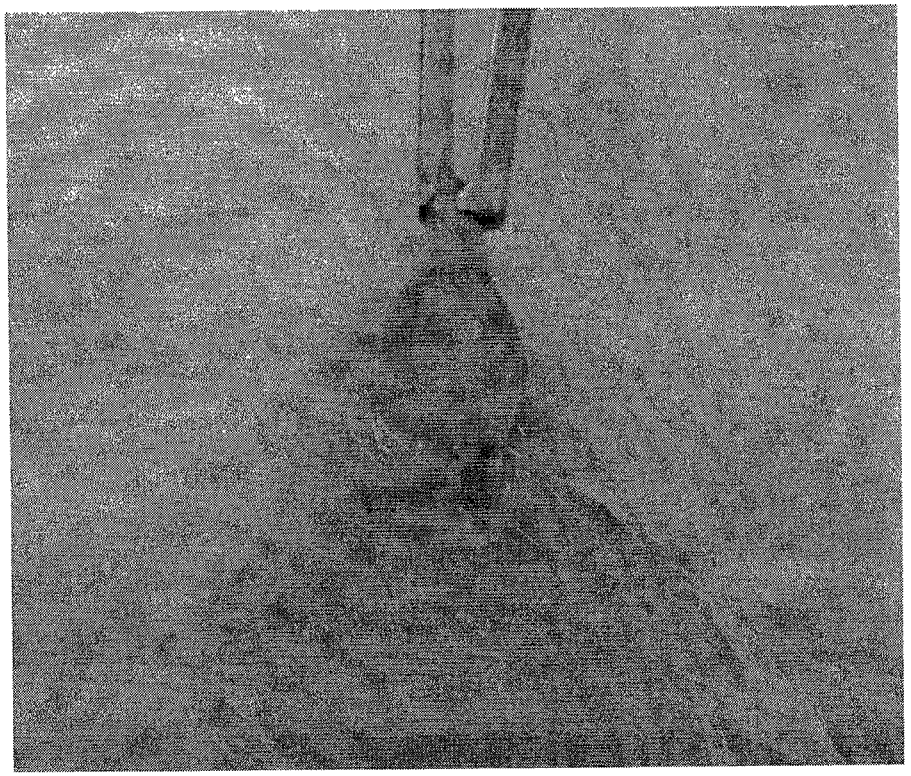
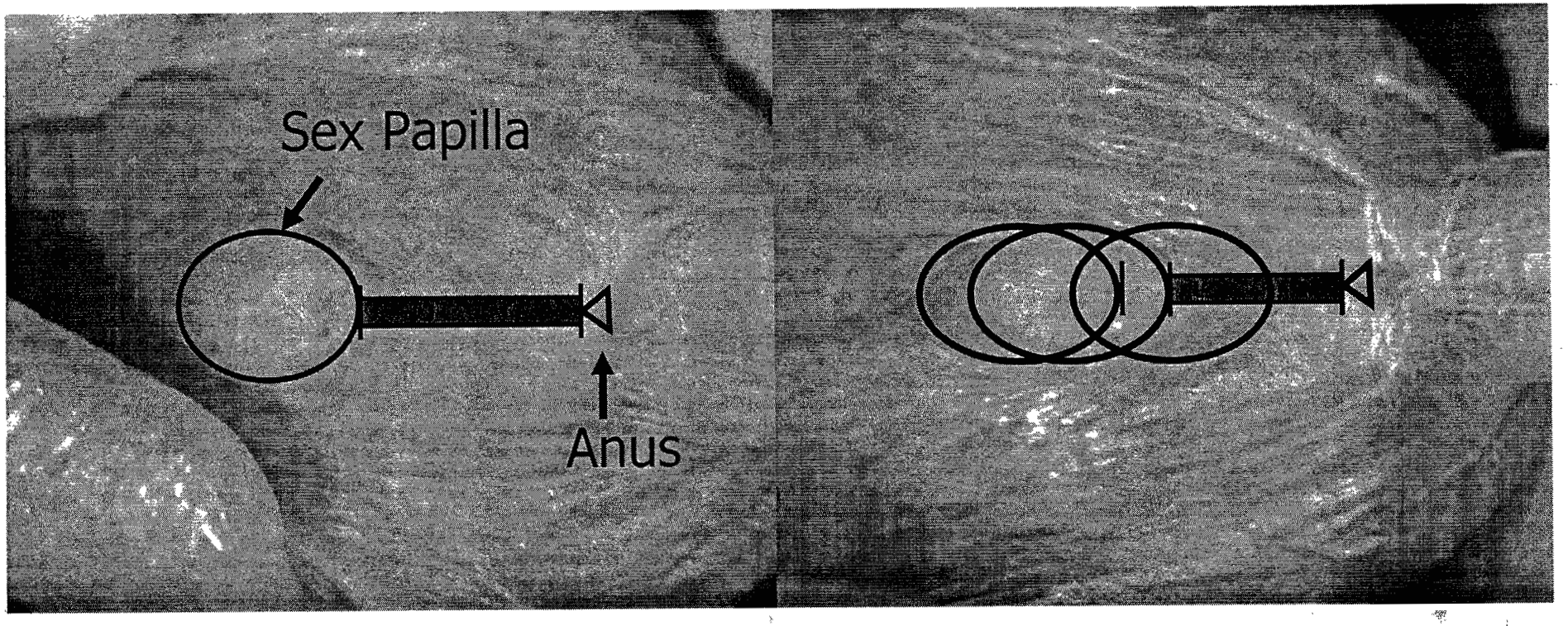


DBP

Barlow & Foster (2003) *Tox Path* **31**: 1-14

Anogenital Distance



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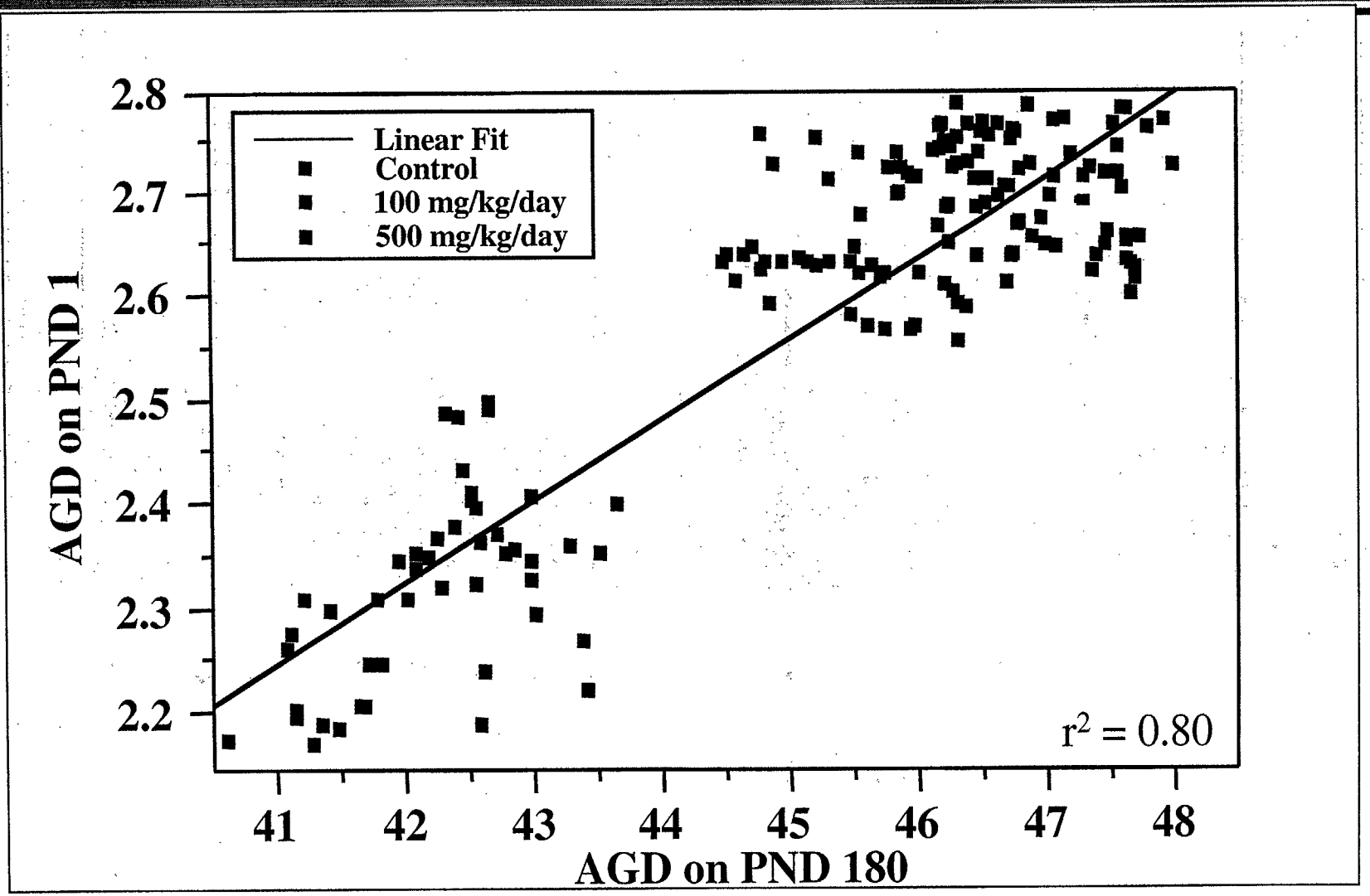
Bivariate Fit of AGD following in utero DBP exposure



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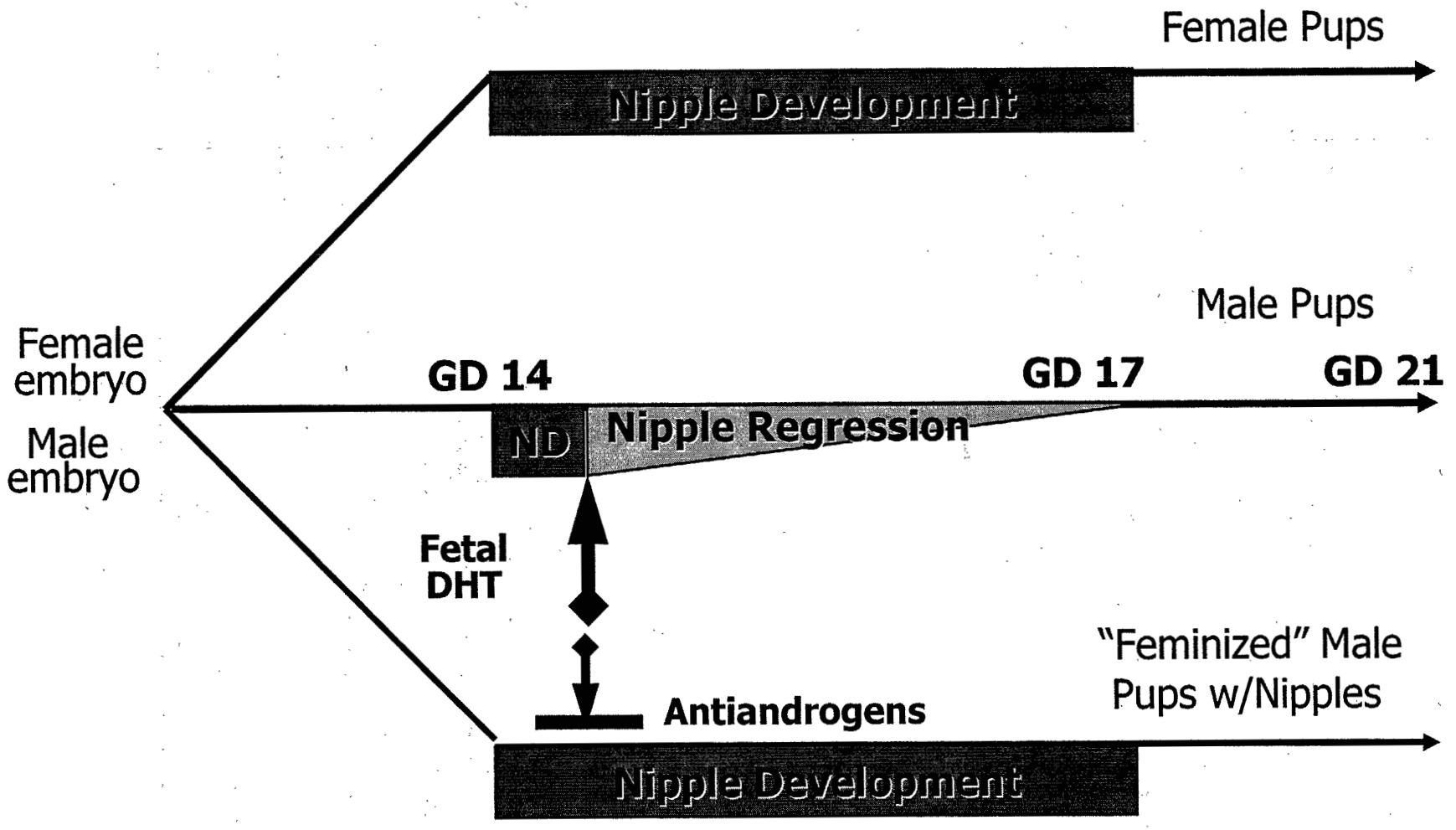
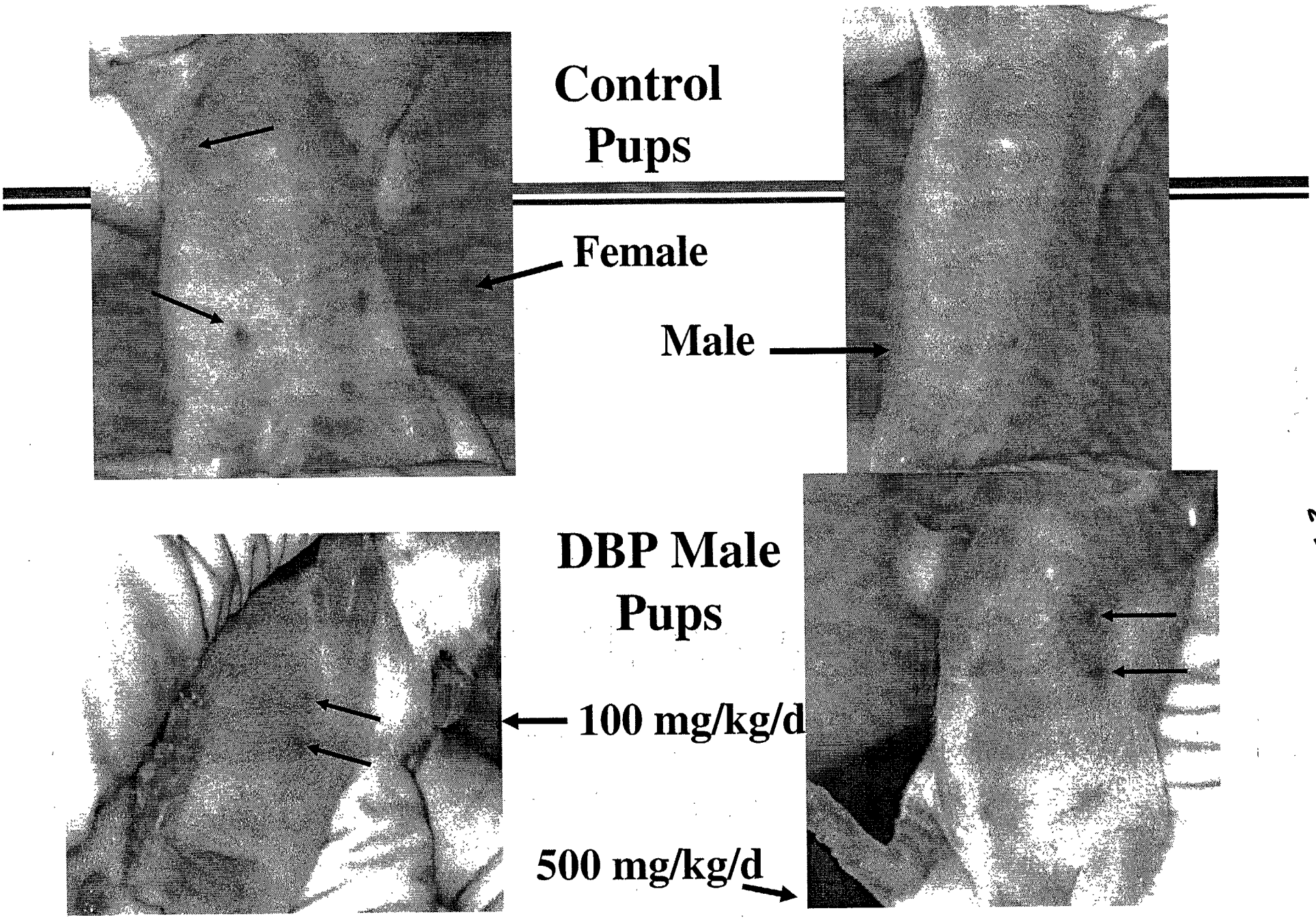
Nipple Development in the Rat





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Mylchreest *et al* (2000), *Tox Sci* **55**:143-151

Transient Vs. Permanent Changes in Phenotype

- There has been some debate on the interpretation of changes in AGD and areolae/ nipples.
  - Indicators of disturbance in androgen status, or
  - True malformations indicative of a rare, but permanent structural change.
- Recent data examining these end points in the same animal at different times (neonatally and adults) has indicated that these changes are likely to be permanent. However a continuum exists with lower dose levels of weaker antiandrogens producing non-statistically significant (transient) changes in adults.

Objectives for Proposed "One-Generation Extension" Study

- Determine whether some of the effects from perinatal exposure to well characterized antiandrogens (di-n-butyl phthalate, vinclozolin) that can be readily detected after puberty are missed in weanling animals of the F_1 generation.
- Determine whether some of these effects occur at an incidence that would go undetected if only 1 male per litter is retained past puberty and examined at adulthood.

ZY

Enhanced Weanling Necropsy

- Far more detailed examination than would normally happen on a multigeneration study.
  - Use of dissecting microscope
  - Significantly greater number of end points
  - Requires very skilled technicians
  - E.g dissection of phallus to determine hypospadias.
  - There are cost and time issues in carrying out such work to be traded off against the more routine adult necropsy that produces greater sensitivity for the same number of animals.

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Selected PND 95 malformations

Text Table 4. F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males	82	95	74	81	74
Epididymis missing	0 (0.0)	0 (0.0)	4 (5.4)	0 (0.0)	33 (44.6)
Reduced in size	1 (1.2)	0 (0.0)	12 (16.2)	0 (0.0)	52 (70.3)
Hypospadias	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
Males with ≥ 1 gubernacular cord	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with ≥ 1 cranial suspensory ligament	0 (0.0)	0 (0.0)	1.0 (1.4)	0 (0.0)	6.2 (8.4)
Phallus, cleft	1 (1.20)	41 (43.2)	74 (100.0)	2.0 (2.5)	26 (35.1)

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Selected PND 21 to 95 comparisons

Text Table 6. Comparison of the Incidence of Male Reproductive System Malformations on pnd 21 Versus pnd 95

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males, pnd 21	74	82	65	71	65
No. males, pnd 95	82	95	74	81	74
Hypospadias:					
Pnd 21	0 (0.0)	8.0 (9.7)	52 (80.0)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
Phallus, cleft:					
Pnd 21	0 (0.0)	4 (4.9)	25 (38.5)	0 (0.0)	2 (3.1)
Pnd 95	1 (1.2)	41 (43.15)	74 (100.0)	2.0 (2.47)	26 (35.14)
Males with >1 gubernuclear wt.[b]					
Pnd 21	74 (100.0)	82 (100.0)	65 (100.0)	69 (97.2)	59 (90.8)
Pnd 95	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with >1 cranial suspensory ligament[b]					
Pnd 21	0 (0.0)	1 (1.32)	0 (0.0)	2.2 (3.1)	2.2 (3.4)
Pnd 95	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	6.2 (8.1)
Vaginal pouch					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	2 (2.4)	43 (58.1)	0 (0.0)	1 (1.4)

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No Prostate effects at PND 21 with Low dose VIN or DBP

Text Table 6. Comparison of the Incidence of Male Reproductive System Malformations on pnd 21 Versus pnd 95

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males, pnd 21	74	82	65	71	65
No. males, pnd 95	82	95	74	81	74
Prostate dorsal lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	31 (32.3)	0 (0.0)	1 (1.5)
Pnd 95	0 (0.0)	0 (0.0)	17 (23.0)	0 (0.0)	3 (4.0)
Prostate dorsal lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	3 (3.2)	20 (27.0)	2 (2.5)	8 (10.8)
Prostate ventral lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	5 (7.7)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	0 (0.0)	12 (16.2)	0 (0.0)	3 (4.0)
Prostate ventral lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	1 (1.2)	7 (7.4)	45 (60.8)	2 (2.5)	8 (10.8)

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Study compromises/ unresolved issues

- No histopathology
  - Difficult to verify some of the milder abnormalities detected by gross examination, particularly low dose DBP and controls. Concordance of response: gross → weight → histology
- Animal numbers /litter and analysis of malformations
  - Real comparison is between what is undertaken normally on a multigen: 3 weanlings+ 1 adult cf all adult offspring (not just 3 as in this case)
  - A statistical comparison between results from 1 adult and 3 adults per litter is desirable.
  - Statistical differences between specific malformations at both ages. Plus a statistical analysis by sample size.

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Study Conclusions - 1

- Dose levels were selected to ensure detection of the agents and therefore effects should be dose-related.
- Some specific male reproductive malformations were detected at PND 95 but not at PND 21(eg vaginal pouch for VIN; prostate malformations for low VIN)
- The incidence of specific malformations detected on PND 95 was greatly increased over the same malformation at PND 21 even though animal numbers were approximately equal.

JD

Flagging of Potential Adverse Effects

Study Type	Reg #	Comments
Teratogenicity	83-3	• When compared with concurrent controls, treated animals show a dose-related increase in malformations (or deaths) on a litter basis in the absence of significant maternal toxicity at the same dose levels.
Reproduction	83-4	• Reproductive effects NOEL less than 100 times the current ADI.

3'

Study Conclusions - 2

- Adverse effects on weights of some organs were more apparent at PND 95 than 21.
- Are the changes in some parameters noted at or before weaning permanent?
  - E.g. Does the lack of a permanent effect on AGD or nipples constitute an adverse response? Does this need to be statistically significant?

Study Conclusions - 3

- "The more males examined per litter, the better the characterization of the litter as responding or not responding adversely to exposure. Enhanced sensitivity with more males examined per litter would increase the likelihood of detection of effects as statistically and biologically significant."
- "For effects with low incidence, such as in the low dose DBP group in this study, the risk with fewer males examined per litter is that the effect might be missed, i.e., the litter would be designated as not responding, on the basis of the one male examined, if that male did not exhibit the effect."